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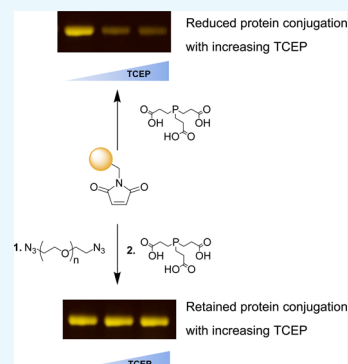
In Situ Quenching of Trialkylphosphine Reducing Agents Using Water-Soluble PEG-Azides Improves Maleimide Conjugation to Proteins

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S Supporting Information

ABSTRACT: Trialkylphosphines tris(2-carboxy-ethyl)-phosphine and tris(3-hydroxypropyl)-phosphine are popular reagents for the reduction of cysteine residues in bioconjugation reactions using maleimides. However, it has been demonstrated that these phosphines are reactive toward maleimide, necessitating their removal before the addition of the Michael acceptor. Here, a method using water-soluble PEG-azides is reported for the quenching of trialkylphosphines in situ, which is demonstrated to improve the level of maleimide conjugation to proteins.



INTRODUCTION

The water-soluble trialkylphosphines, tris(2-carboxy-ethyl)-phosphine **1** (TCEP) and tris(3-hydroxypropyl)-phosphine **2** (THPP), are effective reagents for the reduction of disulfides before performing protein-conjugation reactions using Michael acceptors such as maleimides (Figure 1a).^{1–5} Reactions such as these are important within the pharmaceutical industry for the manufacture of several types of products including antibody–drug conjugates, PEGylated proteins, and conjugate vaccines. Preference for the use of TCEP and THPP over traditional thiol-based reducing agents can be attributed to a number of practical advantages. TCEP and THPP are relatively stable toward aerial oxidation at pH values common for protein conjugations, as well as being nonvolatile and relatively odorless.^{6,7} Importantly, reduction of cysteinyl residues by these phosphines does not result in the formation of mixed disulfides, as is the case with traditional thiols such as dithiothreitol and 2-mercaptoethanol.^{7–10}

Early reports on the use of TCEP in protein conjugation strategies suggested that this phosphine was compatible with maleimide and did not need to be removed before the addition of the Michael acceptor.^{2,11–13} A number of recent reports, however, have confirmed that TCEP and THPP do indeed react with maleimides to reduce conjugation yields significantly.^{8,14–17} Importantly, it has also been demonstrated that ylens formed between maleimide and TCEP, such as **3**, are remarkably stable under physiological conditions and can remain incorporated in the products of some protein conjugations (Figure 1b).¹⁶ As such, it is advantageous to remove the phosphine from the reaction before the addition of maleimide.

A variety of methods are available for the removal of TCEP (e.g., dialysis, TCEP-immobilized resin, column chromatography); however, each has associated drawbacks.¹⁴ As an alternative, Henkel et al. have reported an elegant approach that uses 4-azidobenzoic acid (**4**) to quench excess TCEP through a Staudinger reaction, thus circumventing the need for a purification step when using phosphines in maleimide-based bioconjugations (Figure 1c).¹⁷

In our hands, however, we have found the application of this method to be limited by the low aqueous solubility of **4**, which necessitated increased reaction volumes, thereby reducing the substrate concentrations and reaction rates. Here, we describe the use of azide-modified polyethylene glycols as water-soluble reagents for the quenching of TCEP and THPP in situ to improve yields of protein conjugation reactions using maleimide.

RESULTS AND DISCUSSION

A series of azide-containing ethylene glycols of increasing molecular weights were initially chosen to determine the effect of polymer length on their aqueous solubility and on reactivity toward TCEP. Diazido-ethylene glycols **5–8** are all available commercially or, alternatively, can each be synthesized in good yields following established methods (Scheme 1).

The mass and molar solubilities of PEG-azides **5–8** were determined in a 0.1 M Tris–HCl buffer at pH 7, and all azides were found to be readily soluble in this buffer system with

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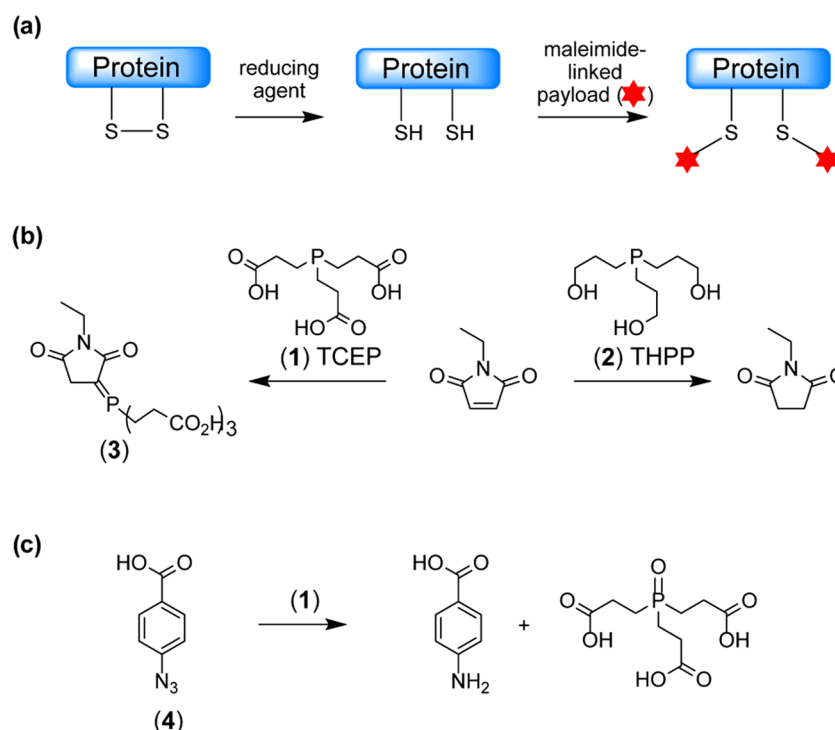
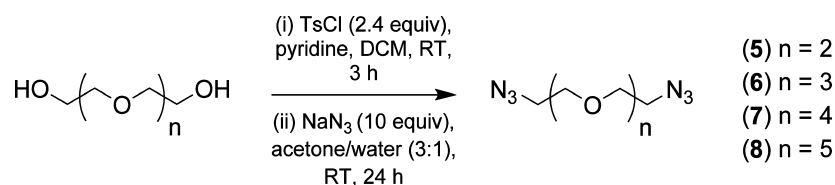


Figure 1. (a) General method for the synthesis of protein conjugates by reduction and conjugate addition to thiols. (b) Reactions of TCEP (1) and THPP (2) with *N*-ethylmaleimide to give the ylene 3 and *N*-ethylsuccinimide, respectively.¹⁶ (c) Oxidation of TCEP using 4-azidobenzoic acid 4 reported by Henkel et al.¹⁷

Scheme 1. Chemical Synthesis of PEG-Azides 5–8



values ranging from 25 mg/mL for the di-PEG 5, up to 130 mg/mL for the penta-PEG 8 (Table 1). By comparison, 4-ABA (4) was found to have a significantly lower mass solubility under similar conditions (Table 1). The solubility of 4 could be increased using different solvent systems, such as 50% aqueous methanol or Tris–HCl buffer at pH 8.0. However, solvent systems such as these are suboptimal for performing maleimide conjugation reactions with proteins.

The ability of azides 5–8 to quench TCEP and THPP by promoting oxidation was then evaluated using ³¹P NMR spectroscopy to quantify the rate of consumption of phosphine.

Table 1. Summary of the Mass and Molar Aqueous Solubilities Measured for PEG-Azides 5–8 and 4-ABA 4

compound	solvent	solubility	
		mg/mL	mM
4	0.1 M Tris–HCl buffer (pH 7.0)	0.5	3
4	0.1 M Tris–HCl buffer (pH 8.0)	4.4	27
4	50% MeOH/water	1.5	9
5	0.1 M Tris–HCl buffer (pH 7.0)	25	125
6	0.1 M Tris–HCl buffer (pH 7.0)	60	246
7	0.1 M Tris–HCl buffer (pH 7.0)	88	304
8	0.1 M Tris–HCl buffer (pH 7.0)	130	356

Solutions of TCEP (25 mM) and PEG-azide (10 equiv) were monitored every 10 min (³¹P NMR), with all azides promoting rapid oxidation of TCEP. Azides 5–7 were found to have similar reactivities, with complete consumption of TCEP occurring after 50–60 min (Figure 2). The penta-PEG azide, 8, was found to react slightly more rapidly, with complete

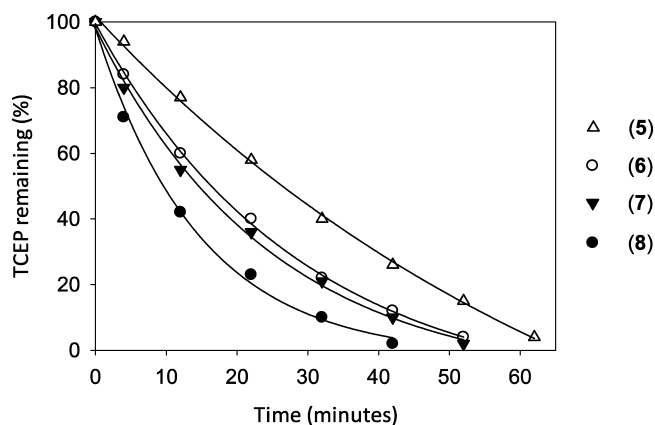


Figure 2. Oxidation rates of TCEP in the presence of PEG-azides 5–8 (250 mM) in a 0.1 M Tris–HCl buffer. Remaining TCEP (%) was determined using ³¹P NMR spectroscopy.

Scheme 2. Reaction of PEG-Azide 7 with *N*-Ethylmaleimide 9 (1.5 equiv) To Give Monotriazole 10 and Ditriazole 11, in 42 and 25% Yields, Respectively

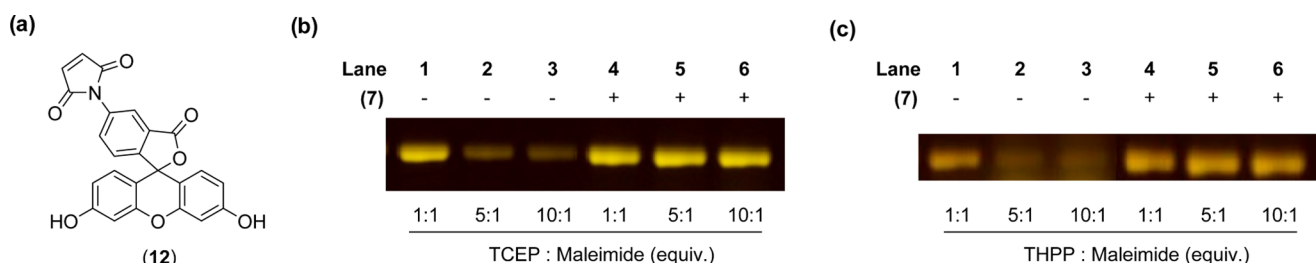
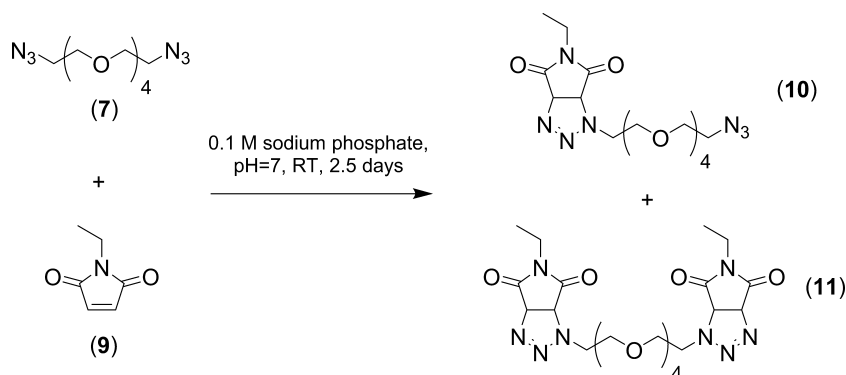


Figure 3. Fluorescent labeling of yeast enolase in the presence or absence of PEG-azide 7. (a) Chemical structure of the fluorescein-maleimide label 12. (b) Lanes 1–3: Yeast enolase (11 μ M) in Tris–HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) was incubated with TCEP (1, 5, or 10 mM) for 45 min (RT) and then treated with 12 (1 mM) for 18 h (37 $^{\circ}$ C). Lanes 4–6: Yeast enolase (11 μ M) in Tris–HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) was treated with TCEP (1, 5, or 10 mM) for 45 min (RT) and then incubated with 7 (100 mM) for 1 h (37 $^{\circ}$ C) before the addition of 12 (1 mM) for 18 h (37 $^{\circ}$ C). (c) Lanes 1–3: Yeast enolase (11 μ M) in Tris–HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) was incubated with THPP (1, 5, or 10 mM) for 45 min (RT) and then treated with 12 (1 mM) for 18 h (37 $^{\circ}$ C). Lanes 4–6: Yeast enolase (11 μ M) in Tris–HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) was treated with THPP (1, 5, or 10 mM) for 45 min (RT) and then incubated with 7 (100 mM) for 1 h (37 $^{\circ}$ C) before the addition of 12 (1 mM) for 18 h (37 $^{\circ}$ C). All protein samples were resolved by SDS-Page (4–12% gradient gel) and fluorescence visualized at 525 nm (Dark Reader).

oxidation of the phosphine occurring after only 40 min. Similarly, all of the azides (5–8) were found to promote the oxidation of THPP under similar conditions to those used for the oxidation of TCEP; however, oxidation occurred at much higher rates and complete consumption of THPP was observed in less than 5 min.

Having established that all of the PEG-azides, 5–8, can effectively promote rapid oxidation of TCEP and THPP under conditions suitable for performing maleimide-based protein conjugation reactions, it was necessary to consider potential side reactions that might occur from their use in situ in a conjugation mixture. In particular, the ability of alkyl azides to react with maleimide to form triazoles is reported in the literature, typically using organic solvents at elevated temperatures.¹⁸ To investigate the potential for PEG-azides to form triazoles with maleimide in an aqueous buffer at an ambient temperature, the tetra-PEG azide, 7, was treated with *N*-ethylmaleimide (9) for 2.5 days, which resulted in the formation of both the monotriazole (10) and ditriazole (11) derivatives isolated in 42 and 25% yields, respectively (Scheme 2). Despite this observation, it was considered that the cross-reaction with maleimide was unlikely to interfere with the use of PEG-azides in situ during protein conjugation strategies given the very slow rate of triazole formation (days) compared to typical rates of maleimide reaction with thiols (min–h).

The use of PEG-azides to improve the yield of bioconjugation reactions was then evaluated in applications in which maleimide is commonly employed as a Michael acceptor.

The functionalization of proteins using maleimide-derivatized fluorescent labels is often used to enable visualization of proteins, with a number of these reagents, for example, *N*-(5-fluoresceinyl)maleimide (12), being commercially available (Figure 3a).^{15,19,20} Tyagarajan et al. have demonstrated previously that the level of labeling of yeast enolase protein (which contains a single internal cysteine and requires denaturation for conjugation to occur) by maleimide-containing fluorescent dyes is significantly diminished when performing the conjugation reaction in the presence of TCEP.¹⁵ We sought to evaluate the impact that the use of PEG-azides in situ would have on the level of fluorescent labeling of yeast enolase using 12. Denatured yeast enolase was treated with varying amounts of TCEP (1, 5, or 10 equiv) and then incubated with 12. In a parallel experiment, TCEP-treated enolase samples were incubated with PEG-azide 7 for 1 h before the addition of compound 12. The fluorescently labeled enolase protein was then resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the level of maleimide conjugation was evaluated by visualization at 525 nm (Dark Reader). Here, the degree of fluorescent labeling of yeast enolase was observed to decrease with increasing amounts of TCEP (Figure 3b, lanes 1–3), consistent with the previous observations of Tyagarajan et al. Significantly, in the presence of both 5 and 10 equiv of TCEP (Figure 3b, lanes 2 and 3, respectively), labeling by maleimide was essentially abolished. However, for enolase samples treated with PEG-azide 7 before the addition of 12, there appeared to be little difference

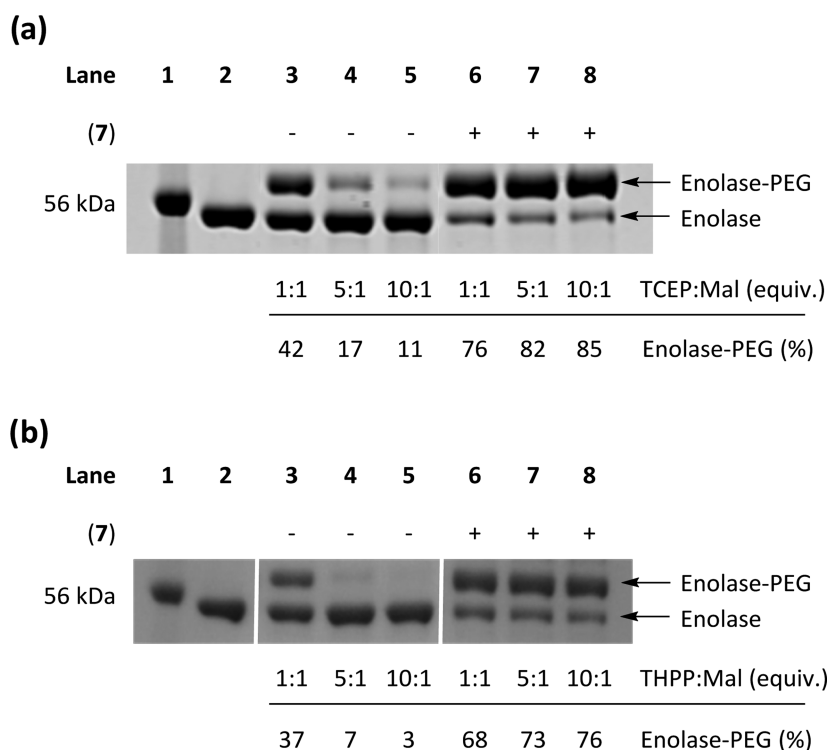


Figure 4. Functionalization of yeast enolase with 2 kDa PEG-maleimide in the presence or absence of 7. (a) Lane 1: protein marker; lane 2: yeast enolase control; lanes 3–5: Yeast enolase (11 μ M) in Tris–HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) was incubated with TCEP (1, 5, or 10 mM) for 45 min (RT) and then treated with 2 kDa PEG-maleimide (1 mM) for 18 h (37 $^{\circ}$ C). Lanes 6–8: Yeast enolase (11 μ M) in Tris–HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) was treated with TCEP (1, 5, or 10 mM) for 45 min (RT) and then incubated with 7 (100 mM) for 1 h (37 $^{\circ}$ C) before the addition of 2 kDa PEG-maleimide (1 mM) for 18 h (37 $^{\circ}$ C). (b) Lane 1: protein marker; lane 2: yeast enolase control; lanes 3–5: Yeast enolase (11 μ M) in Tris–HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) was incubated with THPP (1, 5, or 10 mM) for 45 min (RT) and then treated with 2 kDa PEG-maleimide (1 mM) for 18 h (37 $^{\circ}$ C). Lanes 6–8: Yeast enolase (11 μ M) in Tris–HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) was treated with THPP (1, 5, or 10 mM) for 45 min (RT) and then incubated with 7 (100 mM) for 1 h (37 $^{\circ}$ C) before the addition of 2 kDa PEG-maleimide (1 mM) for 18 h (37 $^{\circ}$ C). All protein samples were resolved by SDS-Page (4–12% gradient gel) and stained using Coomassie blue solution. PEGylated enolase (%) was determined by scanning each band ($n = 3$) using a LI-COR Odyssey CLx and calculating the ratio of PEGylated enolase/total enolase (Image Studio Lite).

between the amount of TCEP used and the extent of fluorescent labeling, with all samples showing significant levels of fluorescence (Figure 3b, lanes 4–6). Similarly, when the above experiment was repeated using THPP as the reducing agent, fluorescent labeling was again seen to decrease significantly with increasing amounts of the phosphine in the absence of azide 7 (Figure 3c, lanes 1–3). In the presence of azide 7, however, the amount of THPP used did not appear to influence the extent of conjugation (Figure 3c, lanes 4–6), consistent with the results observed for TCEP-treated enolase. As such, the preincubation of TCEP- or THPP-treated enolase with PEG-azide 7 before maleimide addition was found to improve the level of fluorescent conjugation regardless of the amount of the phosphine used.

Another important application of maleimide for conjugation to proteins involves the attachment of large PEG polymers (kDa's), a technique used widely within the pharmaceutical industry to improve the pharmacokinetic properties of protein therapeutics. Here, it was considered that investigating the use of azides 5–8 in situ in protein PEGylation reactions would also facilitate the quantification of any improvements to conjugation, as PEGylated protein products could be resolved on the basis of their mass (SDS-PAGE). In addition, the use of excess PEG-maleimide to protein (100 equiv) would identify whether any nonspecific conjugation was occurring, as multiple higher-molecular-weight products would be observed. Dena-

tured yeast enolase protein was reduced with varying amounts of TCEP (1, 5, and 10 equiv) and then incubated with 2 kDa PEG-maleimide. In parallel, samples of the TCEP-reduced enolase were treated with azide 7 for 1 h, before incubation with 2 kDa PEG-maleimide under similar conditions. All samples were then resolved (SDS-PAGE), and the presence of protein was detected by staining with Coomassie Brilliant Blue. To determine the level of protein PEGylation, SDS gels were scanned (LI-COR Odyssey CLx) to quantify the intensity of Coomassie-stained protein bands. Figure 4a shows that the extent of protein PEGylation decreases significantly from 42 to 11% with increasing amounts of TCEP when treated with 2 kDa PEG-maleimide directly following reduction (lanes 3–5). In contrast, for enolase incubated with azide 7 before incubation with 2 kDa PEG-maleimide, increased levels of PEGylation were observed for all samples (76–85%), regardless of the TCEP concentration used (Figure 4a, lanes 6–8). Worthy of mention is that, in the presence of equal molar amounts of maleimide to TCEP, only 42% conjugation is observed following direct treatment with PEG-maleimide (lane 3), whereas the level of conjugation rises to 76% following removal of TCEP by 7 before the addition of maleimide (lane 6). This suggests that the relative rates of reaction between maleimide with either cysteine or TCEP are comparable, consistent with previous observations by Kantner.¹⁶ Similar results were also observed using THPP as the reducing agent.

Treatment of denatured enolase with 2 kDa PEG-maleimide immediately following reduction showed significant levels of unmodified protein (Figure 4b, lanes 3–5), whereas incubation of the reduced protein with 7 before the addition of maleimide resulted in high levels of PEGylation for all concentrations of THPP used (Figure 4b, lanes 6–8). Interestingly, the highest overall levels of protein PEGylation were observed using a 10:1 ratio of the phosphine (TCEP or THPP) when incubated with 7 before the addition of maleimide (Figure 4a, lane 8; and Figure 4b, lane 8). Conversely, the poorest levels of conjugation were seen when using this same 10:1 ratio of the phosphine in the absence of 7 (Figure 4a, lane 3; and Figure 4b, lane 3). This observation could result from more extensive reduction (more thiol present) as a consequence of the ability to use higher TCEP concentrations, given that all excess TCEP is now being removed through the use of PEG-azides.

CONCLUSIONS

In summary, we have shown here that PEG-azides 5–8 have suitable aqueous solubilities to enable their use in bioconjugation strategies employing maleimide and that all of these azides affect the rapid oxidation of both TCEP and THPP. Furthermore, it has been observed that the treatment of phosphine-reduced protein with PEG-azides such as 7 in situ, before the addition of the maleimide, allows for the use of high TCEP concentrations and leads to improved levels of conjugation using both small fluorescent probes and higher-molecular-weight PEGs.

EXPERIMENTAL SECTION

Synthesis of 1,8-Diazido-3,6-dioxaoctane (5). 4-Toluenesulfonyl chloride (3.3 g, 17.3 mmol, 2.6 equiv) was added to a solution of anhydrous pyridine (1.3 g) and triethylene glycol (1.0 g, 6.7 mmol) in anhydrous DCM (10 mL), and the mixture was left to stir under N₂ overnight at room temperature. The solution was then concentrated in vacuo and was subjected to standard work-up (EtOAc). The resultant solid powder was then purified by recrystallization (DCM/petroleum ether) to give the 3,6-dioxaoctane-1,8-ditosylate as a white powder (2.8 g, 92%). NMR spectra were consistent with those reported.²¹ ¹H NMR (400 MHz, CDCl₃): δ 7.78 (d, *J* = 8 Hz, 4H), 7.34–7.31 (m, 4H), 4.14–4.10 (m, 4H), 3.68–3.63 (m, 4H), 3.51 (s, 4H), 2.43 (s, 6H). ¹³C NMR (100 MHz, CDCl₃): δ 144.76, 132.97, 129.76, 127.87, 70.62, 69.12, 68.68, 21.54. HRMS (ESI): Expected for C₂₀H₂₆Na₁O₈S₂ (M + Na⁺) = *m/z* 481.0967. Found: *m/z* 481.0983.

Sodium azide (1.14 g, 17.4 mmol, 4 equiv) was added to a solution of 3,6-dioxaoctane-1,8-ditosylate (2.0 g, 4.3 mmol) in acetone/water (3:1, 24 mL), and the mixture was allowed to stir at 37 °C overnight. The mixture was then concentrated under reduced pressure to remove the acetone, and the product was extracted using ethyl acetate (3 × 20 mL). The organic extract was then washed with saturated brine solution, dried over MgSO₄, concentrated, and then purified by silica gel chromatography (10 → 60% EtOAc/petroleum ether) to give the diazide (5) as a colorless oil (0.70 g, 80%). NMR spectra were consistent with those reported.²¹ ¹H NMR (400 MHz, CDCl₃): δ 3.69–3.67 (m, 8H), 3.37 (t, *J* = 8 Hz, 4H). ¹³C NMR (100 MHz, CDCl₃): δ 70.68, 70.06, 50.64. HRMS (ESI): Expected for C₆H₁₂N₆NaO₂ (M + Na⁺) = *m/z* 223.0919. Found: *m/z* 223.0925.

Synthesis of 1,11-Diazido-3,6,9-trioxaundecane (6). 4-Toluenesulfonyl chloride (2.5 g, 13.4 mmol, 2.6 equiv) was added to a solution of anhydrous pyridine (1 g) and tetraethylene glycol (1.0 g, 5.2 mmol) in anhydrous DCM (10 mL), and the mixture was left to stir under N₂ overnight at room temperature. The solution was then concentrated in vacuo and was subjected to standard work-up (EtOAc). The resultant residue was then purified by silica gel chromatography (10 → 60% EtOAc/petroleum ether) to give 3,6,9-trioxaundecane-1,11-ditosylate as a colorless oil (2.2 g, 85%). NMR spectra were consistent with those reported.²¹ ¹H NMR (400 MHz, CDCl₃): δ 7.74–7.71 (m, 4H), 7.30–7.72 (m, 4H), 4.11–4.08 (m, 4H), 3.62–3.60 (m, 4H), 3.56–3.43 (m, 8H), 2.38 (s, 6H). ¹³C NMR (100 MHz, CDCl₃): δ 144.77, 132.90, 129.77, 127.81, 70.50, 69.26, 68.54, 21.51. HRMS (ESI): Expected for C₂₂H₃₀S₂O₉ (M + Na⁺) = *m/z* 525.1229. Found: *m/z* 525.1279.

Sodium azide (1.04 g, 16.0 mmol, 4 equiv) was added to a solution of 3,6,9-trioxaundecane-1,11-ditosylate (2.0 g, 4.0 mmol) in acetone/water (3:1, 24 mL), and the mixture was allowed to stir at 37 °C overnight. The mixture was then concentrated under reduced pressure to remove the acetone, and the product was extracted using ethyl acetate (3 × 20 mL). The organic extract was then washed with saturated brine solution, dried over MgSO₄, concentrated, and then purified by silica gel chromatography (10 → 60% EtOAc/petroleum ether) to give the diazide (6) as a colorless oil (0.80 g, 82%). NMR spectra were consistent with those reported.²¹ ¹H NMR (400 MHz, CDCl₃): δ 3.67–3.62 (m, 12H), 3.35 (t, *J* = 8 Hz, 4H). ¹³C NMR (100 MHz, CDCl₃): δ 70.63, 70.62, 69.94, 50.59. HRMS (ESI): Expected for C₈H₁₆N₆NaO₃ (M + Na⁺) = *m/z* 267.118159. Found: *m/z* 267.1228.

Synthesis of 1,14-Diazido-3,6,9,12-tetraoxatetradecane (7). 4-Toluenesulfonyl chloride (2.07 g, 10.9 mmol, 2.6 equiv) was added to a solution of anhydrous pyridine (0.8 g) and pentaethylene glycol (1.0 g, 4.2 mmol) in anhydrous DCM (10 mL), and the mixture was left to stir under N₂ overnight at room temperature. The solution was then concentrated in vacuo and was subjected to standard work-up (EtOAc). The resultant residue was then purified by silica gel chromatography (10 → 60% EtOAc/petroleum ether) to give 3,6,9,12-tetraoxatetradecane-1,14-ditosylate as a colorless oil (2.0 g, 87%). NMR spectra were consistent with those reported.²¹ ¹H NMR (400 MHz, CDCl₃): δ 7.78 (d, 4H, *J* = 8 Hz), 7.33 (t, 4H, *J* = 8 Hz), 4.14 (t, 4H, *J* = 8 Hz), 3.68–3.65 (m, 4H), 3.60 (t, 12H, *J* = 8 Hz), 2.43 (s, 6H). ¹³C NMR (100 MHz, CDCl₃): δ 144.71, 133.00, 129.94, 127.83, 70.68, 70.49, 69.81, 68.61, 21.55. HRMS (ESI): Expected for C₂₄H₃₄Na₁O₁₀S₂ (M + H⁺) = *m/z* 547.1672. Found: *m/z* 547.1666.

Sodium azide (0.95 g, 14.6 mmol, 4 equiv) was added to a solution of 3,6,9,12-tetraoxatetradecane-1,14-ditosylate (2.0 g, 3.7 mmol) in acetone/water (3:1, 24 mL), and the mixture was allowed to stir at 37 °C overnight. The mixture was then concentrated under reduced pressure to remove the acetone, and the product was extracted using ethyl acetate (3 × 20 mL). The organic extract was then washed with saturated brine solution, dried over MgSO₄, concentrated, and then purified by silica gel chromatography (10 → 60% EtOAc/petroleum ether) to give the diazide (7) as a colorless oil (0.85 g, 81%). NMR spectra were consistent with those reported.²¹ ¹H NMR (400 MHz, CDCl₃): δ 3.68–3.65 (m, 16H), 3.37 (t, 4H, *J* = 4.0 Hz). ¹³C NMR (100 MHz, CDCl₃): δ 70.65, 70.62, 70.57, 69.95,

50.64. HRMS (ESI): Expected for $C_{10}H_2N_6NaO_4$ ($M + Na^+$) = m/z 311.1443. Found: m/z 311.1434.

Synthesis of 1,17-Diazo-3,6,9,12,15-pentaoxaheptadecane (8). 4-Toluenesulfonyl chloride (1.73 g, 9.1 mmol, 2.6 equiv) was added to a solution of anhydrous pyridine (0.76 g) and hexaethylene glycol (1.0 g, 3.5 mmol) in anhydrous DCM (10 mL), and the mixture was left to stir under N_2 overnight at room temperature. The solution was then concentrated in vacuo and was subjected to standard work-up (EtOAc). The resultant residue was then purified by silica gel chromatography (10 \rightarrow 60% EtOAc/petroleum ether) to give 3,6,9,12,15-pentaoxaheptadecane-1,17-ditosylate as a colorless oil (1.70 g, 82%). NMR spectra were consistent with those reported.²¹ 1H NMR (400 MHz, $CDCl_3$): δ 7.77 (d, 4H, J = 8 Hz), 7.32 (d, 4H, J = 8 Hz), 4.15–4.12 (m, 4H), 3.68–3.55 (m, 20H), 2.42 (s, 6H, Me). ^{13}C NMR (100 MHz, $CDCl_3$): δ 144.70, 133.00, 129.74, 127.89, 70.67, 70.54, 70.49, 70.44, 69.18, 68.60, 21.55. HRMS (ESI): Expected for $C_{26}H_{39}O_{11}S_2$ ($M + H^+$) = m/z 591.1934. Found: m/z 591.1928.

Sodium azide (0.88 g, 13.6 mmol, 4 equiv) was added to a solution of 3,6,9,12,15-pentaoxaheptadecane-1,17-ditosylate (2.0 g, 3.39 mmol) in acetone/water (3:1, 24 mL), and the mixture was allowed to stir at 37 °C overnight. The mixture was then concentrated under reduced pressure to remove the acetone, and the product was extracted using ethyl acetate (3 \times 20 mL). The organic extract was then washed with saturated brine solution, dried over $MgSO_4$, concentrated, and then purified by silica gel chromatography (10 \rightarrow 60% EtOAc/petroleum ether) to give the diazide (8) as a colorless oil (0.89 g, 79%). NMR spectra were consistent with those reported.²¹ 1H NMR (400 MHz, $CDCl_3$): δ 3.67–3.64 (m, 20H), 3.36 (t, J = 4 Hz, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 70.48, 70.44, 69.87, 50.54. HRMS (ESI): Expected for $C_{12}H_{24}N_6NaO_5$ ($M + Na^+$) = m/z 355.1706. Found: m/z 355.1728.

Synthesis of 1-(14-Azido-3,6,9,12-tetraoxatetradec-1-yl)-5-ethyl-3a,6a-dihydropyrrolo[3,4-*d*][1,2,3]-triazole-4,6(1*H*,5*H*)-dione (10) and 1,1'-(3,6,9,12-Tetraoxatetradecane-1,14-diyl)bis(5-ethyl-3a,6a-dihydropyrrolo[3,4-*d*][1,2,3]triazole-4,6(1*H*,5*H*)-dione) (11). 1,14-Diazo-3,6,9,12-tetraoxatetradecane 7 (50.0 mg, 0.173 mmol) and *N*-ethylmaleimide 9 (32.6 mg, 0.260 mmol, 1.5 equiv) were stirred in aqueous sodium phosphate (0.1 M, pH 7.0, 2 mL) at room temperature for 2.5 days. The reaction was diluted with water (20 mL) and extracted with ethyl acetate (2 \times 50 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated. The crude material was purified by silica gel chromatography (10 \rightarrow 60% EtOAc/petroleum ether) to yield 10 (30 mg, 42%) and 11 (23 mg, 25%) as transparent oils. Compound 10: 1H NMR (400 MHz, $CDCl_3$): δ 5.49 (d, J = 11.0 Hz, 1H, $NCOCHNCH_2$), 4.62 (d, J = 11.0 Hz, 1H, $NCOCHNCH_2$), 4.15–4.09 (dt, J = 4.5, 15.1 Hz, 1H, $NCHHCH_2O$), 4.00–3.93 (ddd, J = 3.9, 7.9, 14.8 Hz, 1H, $NCHHCH_2O$), 3.86–3.81 (m, 1H, NCH_2CHHO), 3.78–3.72 (m, 1H, NCH_2CHHO), 3.65–3.55 (m, 14H), 3.50 (q, J = 7.2 Hz, 2H, NCH_2CH_3), 3.36 (t, J = 5.2 Hz, 2H, $CH_2CH_2N_3$), 1.11 (t, J = 7.2 Hz, 3H, CH_2CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.2, 170.6, 81.2, 70.58, 70.54, 70.41, 70.38, 70.32, 70.12, 69.87, 69.1, 58.5, 50.5, 48.1, 34.1, 12.7. HRMS (ESI): Expected for $C_{16}H_{27}N_7NaO_6$ ($M + Na^+$) = m/z 436.1915. Found: m/z 436.1946. IR (thin film): 2107, 1714 cm^{-1} . HPLC: 5 \rightarrow 100% MeCN in H_2O (0.5 mL/min) over 20 min; retention time = 13.50 min, purity = 98.3%. Detection at 254 nm. Compound 11: 1H NMR (400 MHz, $CDCl_3$): δ 5.55 (d, J = 11.0 Hz, 1H, $COCHNCH_2$), 5.54 (d, J =

11.0 Hz, 1H, $COCHNCH_2$), 4.67 (d, J = 11.0 Hz, 1H, $COCHNCH_2$), 4.66 (d, J = 11.0 Hz, 1H, $COCHNCH_2$), 4.19–4.12 (m, 2H, $NCHHCH_2O$), 4.02–3.94 (m, 2H, $NCHHCH_2O$), 3.89–3.83 (m, 2H, NCH_2CHHO), 3.80–3.74 (m, 2H, NCH_2CHHO), 3.68–3.56 (m, 12H), 3.53 (q, J = 7.2 Hz, 4H, NCH_2CH_3), 1.14 (t, J = 7.2 Hz, 6H, CH_2CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.41, 172.39, 170.79, 170.73, 81.34, 81.33, 70.49, 70.36, 70.08, 69.21, 69.06, 58.62, 58.59, 48.14, 48.12, 34.24, 12.76. HRMS (ESI): Expected for $C_{22}H_{34}N_8NaO_8$ ($M + Na^+$) = m/z 561.2392. Found: m/z 561.2365. IR (thin film): 1714 cm^{-1} . HPLC: 5 \rightarrow 100% MeCN in H_2O (0.5 mL/min) over 20 min; retention time = 13.53 min, purity = 99.4%. Detection at 254 nm.

Rates of TCEP Oxidation by PEG-Azides (5–8). Control. The rate of background (aerial) oxidation of TCEP was initially evaluated. A solution of TCEP (100 mM) in Tris–HCl buffer (0.1 M, pH 7) was held at 37 °C, and the mixture was analyzed at 3 and 63 min using ^{31}P NMR spectroscopy to quantify the levels of phosphine oxide present. No oxidation of TCEP was observed under these conditions (Figure S1).

PEG-Azide-Promoted Oxidation of TCEP. A solution of TCEP–HCl (14 mg, 50 μ mol) and PEG-azide (500 μ mol, 10 equiv) in 0.1 M Tris–HCl buffer (2 mL, pH 7) was held at 37 °C, and the solution was analyzed by ^{31}P NMR spectroscopy at times 3, 13, 23, 33, 43, 53, and 63 min. The level of TCEP oxidation was calculated by comparison of integrals corresponding to the phosphine and phosphine oxide. It should be noted that PEG-azide 5 was only partially soluble under these conditions.

PEG-Azide-Promoted Oxidation of THPP. A solution of THPP (10 mg, 48 μ mol) and PEG-azide 8 (40 mg, 120 μ mol, 2.5 equiv) in 0.1 M Tris–HCl buffer (2 mL, pH 7) was held at 37 °C, and the solution was analyzed by ^{31}P NMR spectroscopy at time 3 min. Complete conversion to the phosphine oxide was observed at this time as no signal corresponding to the remaining phosphine was present.

Fluorescent Labeling of Yeast Enolase in the Absence/Presence of PEG-Azide (7). Denaturation of Yeast Enolase Protein. A solution of yeast enolase (1 mg/mL) in deoxygenated Tris–HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) containing 8 M urea was heated to 85 °C and held at this temperature for 15 min. The solution was allowed to cool to room temperature before use in subsequent experiments.

Labeling of Yeast Enolase with Maleimide Fluorescein (11) Following Reduction with TCEP or THPP. Aliquots (100 μ L) of denatured yeast enolase (1 mg/mL, 11 μ M) were treated with varying concentrations of TCEP or THPP (1–10 mM) and incubated for 45 min at 25 °C. Maleimide fluorescein 11 (1 mM) was subsequently added to the enolase solutions at each phosphine concentration and incubated at 37 °C for 18 h. Samples (15 μ L) were taken from each of the reactions and added to the Laemmli sample buffer (15 μ L) and heated (85 °C, 8 min). Aliquots (9 μ L) of these solutions were then loaded into a precast gradient gel (4–12% Bis-Tris, Invitrogen) along with a protein ladder (EZ-Run, Fisher Scientific) and resolved by SDS Page electrophoresis [MOPS running buffer (Invitrogen), 180 V, 60 min]. The precast gels were first stained by Coomassie solution and destained using a water/ethanol/acetic acid (16:3:1) solution to confirm equal protein loading. Fluorescence was then visualized at 525 nm (Dark Reader).

Labeling of Yeast Enolase with Maleimide Fluorescein (11) Following Quenching of TCEP or THPP with PEG-Azide (7). Aliquots (100 μ L) of denatured yeast enolase (1 mg/mL,

11 μM) were treated with varying concentrations of TCEP or THPP (1–10 mM) and incubated for 45 min at 25 $^{\circ}\text{C}$. Samples were then treated with PEG-azide 7 (100 mM) and held for 1 hour at 37 $^{\circ}\text{C}$. Maleimide-fluorescein (1 mM) was subsequently added to these solutions and incubated at 37 $^{\circ}\text{C}$ for 18 h. Samples (15 μL) were taken from each of the reactions and added to the Laemmli sample buffer (15 μL) and heated (85 $^{\circ}\text{C}$, 8 min). Aliquots (9 μL) of these solutions were loaded into a precast gradient gel (4–12% Bis-Tris, Invitrogen) along with a protein ladder (EZ-Run, Fisher Scientific) and resolved by SDS Page electrophoresis [MOPS running buffer (Invitrogen), 180 V, 60 min]. The precast gels were stained by the Coomassie solution and destained using a water/ethanol/acetic acid (16:3:1) solution to confirm equal protein loading. Fluorescence was then visualized at 525 nm (Dark Reader).

PEGylation of Yeast Enolase in the Absence/Presence of Azide (7) Following Reduction by TCEP or THPP. Labeling of Yeast Enolase with 2 kDa-PEG Maleimide Following Reduction with TCEP or THPP. Aliquots (100 μL) of denatured yeast enolase (1 mg/mL, 11 μM) were treated with varying concentrations of TCEP or THPP (1–10 mM) and incubated for 45 min at 25 $^{\circ}\text{C}$. 2 kDa-PEG maleimide (1 mM) was subsequently added to the enolase solutions at each phosphine concentration and incubated at 37 $^{\circ}\text{C}$ for 18 h. Samples (15 μL) were taken from each of the reactions and added to the Laemmli sample buffer (15 μL) and heated (85 $^{\circ}\text{C}$, 8 min). Aliquots (9 μL) of these solutions were loaded into a precast gradient gel (4–12% Bis-Tris, Invitrogen) along with a protein ladder (EZ-Run, Fisher Scientific) and resolved by SDS Page electrophoresis [MOPS running buffer (Invitrogen), 180 V, 60 min]. The precast gels were stained by the Coomassie solution and destained using a water/ethanol/acetic acid (16:3:1) solution. The gel was then scanned using a LI-COR Odyssey CLx to quantify PEGylated enolase (Image Studio Lite).

Labeling of Yeast Enolase with 2 kDa-PEG Maleimide Following Quenching of TCEP or THPP with PEG-Azide (7). Aliquots (100 μL) of denatured yeast enolase (1 mg/mL, 11 μM) were treated with varying concentrations of TCEP or THPP (1–10 mM) and incubated for 45 min at 25 $^{\circ}\text{C}$. Samples were then treated with PEG-azide 7 (100 mM) and held for 1 hour at 37 $^{\circ}\text{C}$. 2 kDa-PEG maleimide (1 mM) was subsequently added to these solutions and incubated at 37 $^{\circ}\text{C}$ for 18 h. Samples (15 μL) were taken from each of the reactions and added to the Laemmli sample buffer (15 μL) and heated (85 $^{\circ}\text{C}$, 8 min). Aliquots (9 μL) of these solutions were loaded into a precast gradient gel (4–12% Bis-Tris, Invitrogen) along with a protein ladder (EZ-Run, Fisher Scientific) and resolved by SDS Page electrophoresis [MOPS running buffer (Invitrogen), 180 V, 60 min]. The precast gels were stained by the Coomassie solution and destained using a water/ethanol/acetic acid (16:3:1) solution. The gel was then scanned using a LI-COR Odyssey CLx to quantify PEGylated enolase (Image Studio Lite).

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b01094.

Supplemental figures as described in the text; ^1H NMR and ^{13}C NMR spectra, HRMS, IR spectra, and HPLC chromatograms for compounds 10 and 11 (PDF)

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Notes

The authors declare no competing financial interest.

Data access statement: All data supporting this study are provided in the Results section of this paper or in the Supporting Information accompanying this paper.

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